

Patent  
Attorney's Docket No. 033072-026

(Sequence ID No.: 55) shows the PCR amplified human V2R DNA fragment, incorporating the changes introduced in the PCR primers.

**[0061]** Figure 4A is a schematic of the pEArrB-1 vector resulting from digestion of the PCR-amplified V2R DNA fragment and cloning into the pcDNA3.1zeo+ vector. Figure 4B is a schematic of the pEArrB-1/GPCR vector resulting from the insertion of the nucleic acids of the GPCR of interest (after PCR-amplification and digestion of the nucleic acids of the GPCR) into the pEArrB-1 vector. Figure 4C is the sequence (Sequence ID No.: 56) of the 31 amino acid peptide that will be the carboxy terminus of the modified GPCR. The first two amino acids will be alanine residues, and the last 29 amino acids will be from the V2R carboxyl terminus (V2R amino acids 343 - 371).

**[0065]** Figure 8A (Sequence ID Nos.: 57 - 65) shows the amino acid composition of the carboxyl-terminal tails of the V2R,  $\beta_2$ AR, and various mutant receptors beginning with the putative sites of palmitoylation in bold (Cys-342 for the V2R constructs 1-5, and Cys-341 for the  $\beta_2$ AR constructs 6 - 9). Underlined are the mutations made by alanine substitution and the last 10 amino acids of the V2R (Sequence ID Nos.: 66 - 68) tail when added to the  $\beta_2$ AR. Figure 8B shows the carboxyl-terminal tails of the NTR-1 (Sequence ID Nos.: 69 - 71), OTR (Sequence ID Nos.: 72 - 75), and SPR (Sequence ID Nos.: 76 - 81) which contain multiple clusters of serine and threonine residues. Receptor mutants resulting from the mutation of individual clusters to alanine residues are indicated below each wild-type receptor.

**[00259]** Construction of plasmids containing the hemagglutinin epitope (HA)-tagged  $\beta_2$ AR,  $\beta$ arr2-GFP,  $\beta$ arr1-GFP,  $\beta$ -arrestin1, and  $\beta$ -arrestin2 as described in Ferguson, S. S., Downey, W. E., 3<sup>rd</sup>, Colapietro, A. M., Barak, L. S., Menard, L., and Caron, M. G. (1996) *Science*, 271:363-366; Zhang, J., Barak, L. S., Anborgh, P. H., Laporte, S. A., Caron, M. G., and Ferguson, S. S. (1999) *J. Biol. Chem.*, 274:10999-11006; Barak, L. S., Tiberi, M., Freedman, N. J., Kwatra, M. M., Lefkowitz, R. J., and Caron, M. G. (1994) *J. Biol. Chem.*, 269:2790-2795; Barak, L. S., Ferguson, S. S., Zhang, J., and Caron, M. G. (1997) *J. Biol. Chem.*, 272:27497-27500.

**[00280]** Whole cell cyclase assays were performed on transfected HEK-293 cells using varying concentrations of isoproterenol ( $1 \times 10^{-12}$  M to  $1 \times 10^{-5}$  M) or AVP ( $1 \times 10^{-12}$  M to  $1 \times$

10<sup>-5</sup> M) as described in Zhang, J., Barak, L. S., Winkler, K. E., Caron, M. G., and Ferguson, S. S. G. (1997) *J. Biol. Chem.*, 272:27005-27014. For membrane adenylyl cyclase assays, transfected HEK-293 cells were harvested by scraping in ice-cold lysis buffer (10 mM Tris-HCl, 5 mM EDTA, pH = 7.4) and membranes were prepared by disruption with a Polytron homogenizer for 20 s at 20,000 rpm followed by centrifugation at 40,000 x g. The cell membrane was resuspended in lysis buffer by Polytron homogenization for 15 s at 20,000 rpm, centrifuged, and resuspended in ice-cold assay buffer (75 mM Tris-HCl, 2 mM EDTA, 15 mM MgCl<sub>2</sub>, pH = 7.4) to a final concentration of 1-2 µg/µl membrane protein. Equivalent amounts of membrane protein in 20-µl aliquots, were assayed for agonist-stimulated adenylyl cyclase activity in a final volume of 50 µl as described in Zhang, J. et al. *J. Biol. Chem.*, 272:27005-27014.

#### REMARKS

The specification has been amended merely formally to place the application in better form for examination. No new matter has been added. Favorable consideration is respectfully solicited.

The Examiner is invited to contact the undersigned at the below-listed telephone number, if it is believed that prosecution of this application may be assisted thereby.

Respectfully submitted,

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**Attachment to Preliminary Amendment dated March 20, 2002**

Marked-up Copy

Paragraphs [0001], [0059] - [0061], [0065], [00259], and [00280]

[0001] This application claims priority under 35 U.S.C § 120 to U.S.S.N. 60/245,772, filed November 3, 2000 and U.S.S.N. 60/260,363, filed January 8, 2001, the contents of which are incorporated by reference in their entirety. This invention was made with Government support under Grant Nos. HL61365 and NS19576 awarded by the National Institutes of Health. The Government has certain rights in the invention.

[0059] Figure 2 is an illustrative, non-limiting list (Sequence ID Nos.: 15 - 53) of known receptors, including the amino acid sequence for their carboxyl terminal tails and appropriate classification. For the Class B receptor examples, the residues that may function as phosphorylation sites in the enhanced affinity motifs are shown in bolded italics.

[0060] Figure 3A shows human V2R nucleic acids (Sequence ID No.: 54) encoding the last 29 amino acids of the human V2R carboxyl terminus and the adjacent stop codon. Figure 3B (Sequence ID No.: 55) shows the PCR amplified human V2R DNA fragment, incorporating the changes introduced in the PCR primers.

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